Rahul Bhambure[1]
Mahesh Bule[1]
Nikhil Shaligram[1]
Madhusudan Kamat[1]
Rekha Singhal[1]

[1] Department of Food Engineering and Technology, Institute of Chemical Technology, Matunga, Mumbai, India.

Research Article

Extracellular Biosynthesis of Gold Nanoparticles using *Aspergillus niger* – its Characterization and Stability

The development of an eco-friendly and reliable process for the synthesis of gold nanomaterials using microorganisms is gaining importance in the field of nanotechnology. In the present study, gold nanoparticles have been synthesized by reduction of aqueous gold ions using the culture supernatant of *Aspergillus niger* NCIM 616. The synthesis of the gold nanoparticles was monitored by UV-visible spectroscopy. The particles thereby obtained were characterized by UV, Fourier transform infrared (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and X-ray diffraction (XRD). The stability of the synthesized gold nanoparticles was analyzed by zeta potential measurement. Treatment of the fungal supernatant with aqueous Au^{+} ions produced nanoparticles with an average particle size of 12.79 ± 5.61 nm. Different characterization studies showed that the extracellular enzyme secreted by *Aspergillus niger* NCIM 616 might be responsible for both formation and capping of the metal nanoparticles.

Keywords: *Aspergillus niger*, Biological synthesis, Biosynthesis, Gold nanoparticles, Nanoparticles, Nanoparticle stability

Received: December 10, 2008; *revised:* April 3, 2009; *accepted:* April 7, 2009

DOI: 10.1002/ceat.200800647

1 Introduction

A key aspect of nanotechnology concerns the development of reliable experimental protocols for the synthesis of nanomaterials over a range of chemical compositions, sizes, and high monodispersity. In the context of the current drive to develop eco-friendly processes and green technologies in material synthesis, the approach of biological synthesis of nanoparticles assumes considerable importance [1]. The impetus of synthesis has shifted from physical and chemical processes towards 'green' chemistry and bioprocesses [2]. The use of microorganisms in the synthesis of nanoparticles is a relatively recent, new, and exciting area of research with considerable potential for development [3]. Microorganisms like yeast, bacteria, and fungi play an important role in the remediation of toxic metals by reducing them under stress conditions which forms the basis for the use of microorganisms in the biosynthesis of nanoparticles [4]. Such microorganisms have recently been recognized as possible eco-friendly nanofactories [5]. Some examples of nanoparticle formation by organisms are magnetotactic bacteria synthesizing magnetite nanoparticles [6] and S-layer bacteria producing gypsum and calcium carbonate layers [7]. Sastry et al. [1] reported the reduction of gold and silver ions to respective metallic nanoparticles when exposed to the fungi *Verticillium* sp. and *Fusarium oxysporum*. Proteins and other biomacromolecules control the nucleation and growth of these inorganic structures [8, 9]. Various microbes are known to reduce metal ions to the metals [1, 10, 11]. The enzymes in the viable microbes catalyze the active metal transformation processes. The microorganisms probably play a role in providing a multitude of nucleation centers and establish conditions for obtaining highly disperse nanoparticle systems. They slow down or entirely prevent aggregation by immobilizing the particles and providing a viscous medium [12].

Gold nanospheres show plasmon resonance with absorption at nearly 520 nm. When light is passed through the solution of gold nanoparticles, a portion of the incident light is scattered but the absorbed light heats the particles and increases the localized temperature. This is exploited in various proposed photo-thermal therapeutic applications of gold nanoparticles [13]. Some attempts have been made for extracellular biosynthesis of gold nanoparticles using different strains of bacte-

Correspondence: Prof. R. S. Singhal (rekha@udct.org), Department of Food Engineering and Technology, Institute of Chemical Technology, Matunga, Mumbai – 400 019, India.

ria like *Pseudomonas aeruginosa* [14], *Bacillus subtilis* 168 [5, 15], extremophilic actinomycetes like *Thermomonospora* sp. [16], and fungi like *Verticillium* sp. [17], and *Fusarium oxysporum* [18].

This study demonstrates the extracellular biosynthesis of stable gold nanoparticles using the fungus *Aspergillus niger*. We have used this fungus previously to study fermentative production of squalene in submerged fermentation [19], and the supernatant of the seed media obtained after separating the cells have been used for the synthesis of gold nanoparticles.

2 Materials and Methods

2.1 Media Components

Glucose, yeast extract, peptone for bacteriology, malt extract, agar, and auric chloride AR grade were purchased from Himedia Ltd, Mumbai, India.

2.2 Microorganisms and Culture Conditions

The fungus *Aspergillus niger* NCIM 616 was obtained from the National Collection of Industrial Microorganisms (NCIM), NCL, Pune and maintained on potato-dextrose agar slants at 25 °C. Stock cultures were maintained by subculturing at monthly intervals. The fungus was grown at pH 5.5 and 25 °C for 7 days.

2.3 Production of Fungal Biomass Using the Fungus *Aspergillus niger* NCIM 616

Aspergillus niger NCIM 616 was cultivated in a media containing 3.0 g L^{-1} malt extract, 3.0 g L^{-1} yeast extract, 5.0 g L^{-1} peptone, and 10 g L^{-1} D-glucose in distilled water [20]. The initial pH of the culture was adjusted to 5.5 before autoclaving. Spore suspensions containing 10^7 spores per mL of *Aspergillus niger* NCIM 616 were inoculated from 7 days old agar slants into 500 mL Erlenmeyer flasks containing 100 mL of sterile medium. The flasks were kept on a rotary shaker (180 rpm) at 28 ± 2 °C for 96 h.

The mycelial mass of *Aspergillus niger* NCIM 616 was then separated from the culture broth by centrifugation (5000 rpm) at 15 °C for 20 min and the settled mycelia were washed thrice with Milli-Q deionized water. The washed mycelia were then added in deionized water and incubated on a rotary shaker (180 rpm) at 28 ± 2 °C for 72 h. After 72 h, the biomass was separated by filtration and the aqueous filtrate was used for the biosynthesis of nanoparticles.

2.4 Biosynthesis of Gold Nanoparticles

Fifty milliliter of the aqueous filtrate was added to 50 mL of 2 mM auric chloride solution to make the final auric chloride concentration to 1 mM in a 500 mL flask. The solution was then kept on a rotary shaker (180 rpm) at 28 ± 2 °C for 96 h. Simultaneously, a control of only aqueous filtrate and a control containing only auric chloride solution were maintained under the same conditions.

2.5 Characterization of Gold Nanoparticles

The formation of gold nanoparticles was monitored by visual inspection of the solution, as well as by periodical recording of the UV-vis spectra of the reaction mixture. The UV-vis spectroscopy measurements were recorded on a UV visible 1650 PC Shimadzu double beam spectrophotometer (France). The aqueous filtrate containing gold nanoparticles was freeze-dried and used for further characterization. A laboratory-scale vacuum freeze dryer (Ref-Vac Consultancy, India) was used for this study. It consisted of a cylindrical stainless-steel drying chamber, a condenser capable of operating at –35 °C, and a vacuum pump (Crompton Greaves, India). Samples were frozen in an external deep freezer (Blue Star, India) at –23 °C for 6 h. Meanwhile, the freeze dryer was started and the temperature of the condenser was allowed to reach its minimum level of –35 °C. Thereafter, the frozen sample was placed in the drying chamber, and subsequently the vacuum pump and the heater were started. The temperature of the sample and pressure inside the chamber were set at 25 °C and 30 Pa, respectively.

Scanning electron microscopy (SEM) of the freeze-dried sample was performed by mounting nanoparticles on specimen stubs with double-sided adhesive tape, coated with platinum in a sputter coater, and examined under JEOL 6386® SEM (Japan) at 10 kV. For transmission electron microscopy (TEM), a drop of aqueous solution containing the gold nanomaterials was placed on the carbon-coated copper grids and dried under an infrared lamp. Micrographs were obtained using a Philips® CM 200 operating at 200 kV (USA). To determine the Fourier transform infrared (FTIR) pattern of the sample, the fungal filtrate containing the gold nanoparticles was freeze-dried and the dried powder was diluted with potassium bromide at a ratio of 1:100 and the spectrum was recorded in an Perkin Elmer FTIR Spectrum BX (Wellesley, MA, USA). The freeze-dried reaction mixture embedded with the gold nanoparticles was used for X-ray diffraction (XRD) analysis. The XRD pattern was recorded on an X'Pert Pro from PANalytical (USA) operating at 40 kV and a current of 30 mA with Cu K_a radiation ($\lambda = 1.54$ °A). Zeta potential measurements of nanosuspensions were performed using a Delsa™ Nano Beckman Coulter (USA).

3 Results and Discussion

This work explores the biosynthesis of gold nanoparticles using *Aspergillus niger* NCIM 616 which was previously used for squalene production. The synthesis of gold nanoparticles was validated by visually monitoring three flasks containing only the aqueous filtrate prepared from *Aspergillus niger* NCIM 616 fermentation, auric chloride solution, and reaction mixture of the aqueous filtrate with auric chloride. The fungal aqueous filtrate and the auric chloride solution were observed to retain their original color, whereas the auric chloride mixed

supernatant turned to dark purple (ruby red) after 24 h of incubation (see Fig. 1). The appearance of a dark purple color in solution is a clear indication of the formation of gold nanoparticles in the reaction mixture. The change in color of the solution is due to the excitation of surface plasmon vibrations in the gold nanoparticles, which is the characteristic property of the nanoparticles [21].

The UV-vis spectra of the aqueous reaction mixture were recorded (see Fig. 2). Aliquots of the reaction mixture were withdrawn at various time intervals and scanned on a UV-visible spectrometer. A strong surface plasmon resonance was centered at 540 nm. This is a characteristic property of colloidal gold [17]. The spectra clearly showed an increase in the intensity of absorbance of the gold nanosuspension with time (see Fig. 2b), indicating the formation of an increased number of gold nanoparticles in the solution with the progress of the reaction [22]. Over a period of 96 h, the gold nanoparticle peak remained close to 540 nm, indicating the particles to be well dispersed in solution. Aggregation was also observed. Ahmad et al. [23] have reported a very good monodispersity of gold nanoparticles synthesized by *Thermonospora* sp. The UV-visible spectra in lower wavelength regions (200 – 400 nm) show the absorption of UV light near to 280 nm, suggesting the presence of protein in solution and aromatic amino acids in these proteins to be responsible for absorbance.

SEM determination of the freeze-dried sample showed formation of gold nanoparticles (see Fig. 3). The morphology of the nanoparticles was highly variable. Further insight into the morphology and size details was provided by TEM analysis. Fig. 4 of TEM showed all the nanoparticles to be well separated without agglomeration. The morphology of the nanoparticles was uniform, with spherical and occasionally elliptical nanoparticles being observed in the micrograph. The separation of the gold nanoparticles observed in the TEM image could be due to capping by proteins which may play a role in the stabilization of the gold nanosuspension. The particle size of the gold nanoparticles in the reaction mixture was confirmed by the TEM image (see Fig. 5) which showed an average particle size of 12.79 ± 5.61 nm.

The amide linkages between amino acid residues in proteins give rise to well-known characteristic bands in the infrared region of the electromagnetic spectrum. Fig. 6 shows the FTIR spectrum of the freeze-dried powder of gold nanoparticles formed after 96 h of incubation with the aqueous filtrate obtained from the fungus. The FTIR measurement indicated that the structure of proteins was not affected because of its interaction with Au^+ ions or nanoparticles. The bands seen at 3217 cm^{-1} correspond to the stretching vibrations of primary amines and corresponding bending vibrations were seen at 1645 cm^{-1}. The two bands observed at 1383 and 1112 cm^{-1} can be assigned to the C–N stretching vibrations of aromatic and aliphatic amines, respectively. The presence of amide linkages suggests that there are some proteins in the reaction mixture. These proteins might be responsible for the formation of the nanoparticles and may play an important role in the stabilization of the formed nanoparticles.

One of the important ways to characterize nanoparticles is by evaluating the XRD spectrum of the sample (see Fig. 7). The XRD spectrum of the nanoparticles synthesized in this work confirmed the presence of crystalline gold nanoparticles (Joint Committee on Powder Diffraction Standards file no. 04-0784). The intense peaks observed agreed with Bragg's reflection of gold nanoparticles reported in the literature [24]. The gold nanoparticles synthesized earlier using bacteria [5, 14, 15], fungi [17, 18], actenomycetes [23], and plant extract [25] also validate our current finding of biosynthesis of gold nanoparticles.

The zeta potential measurement of the gold nanosuspension was performed using a Delsa^{TM} Nano Beckman Coulter apparatus. The mean value of zeta potential –32.10 mV (see Tab. 1) indicated the gold nanosuspension to be stable without any aggregation (see Fig. 8). The prevention of aggregation could be due to the protein, which acts as a capping agent on the nanoparticles. It has been reported earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes present in the cell wall of mycelia. Therefore, stabilization of the gold nanoparticles by protein is a possibility [26].

The potential applications of gold nanoparticles are in the field of catalysis [27–29], biomedicine [30–33], and

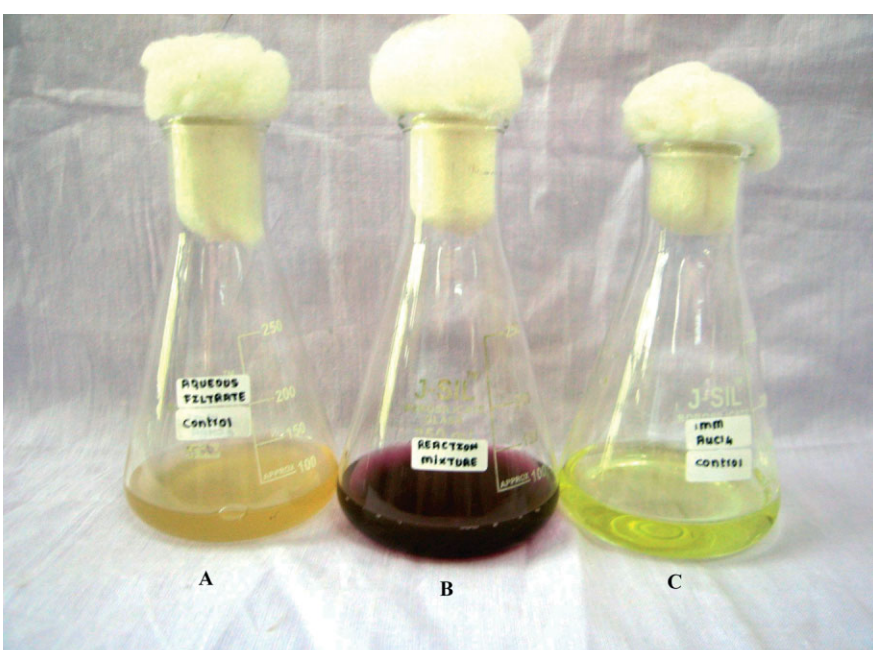

Figure 1. Visual observation of the gold nanoparticle formations: (A) aqueous filtrate control, (B) reaction mixture, (C) auric chloride control.

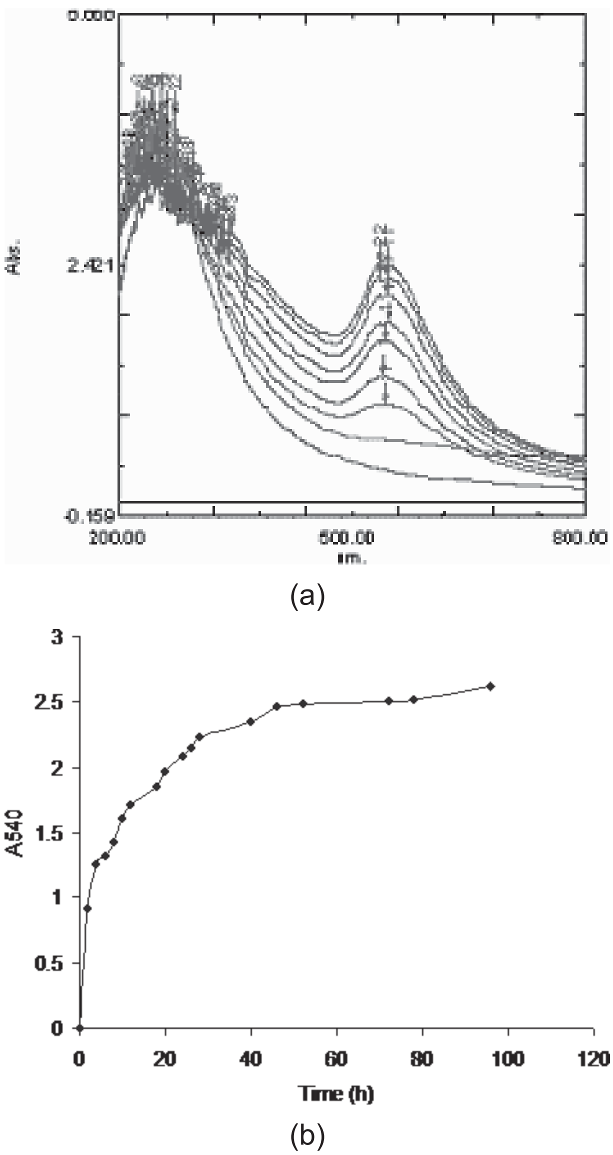

Figure 2. (a) UV-vis spectra recorded with respect to time after the reaction of 2 mM auric chloride solution with aqueous filtrate obtained from *A. niger.* (b) Increase in the absorbance with respect to time after the reaction of 2 mM auric chloride solution with aqueous filtrate obtained from *A. niger.*

photo-electronic materials [35, 35]. This process of extracellular biosynthesis of gold nanoparticles using the fungal system is advantageous in homogeneous catalysis and nonlinear optics. It is free from any toxic chemicals or solvents. This is impossible when nanoparticles are bound to cell biomass. It is eco-friendly and also lends itself readily for large-scale production. In general, biological systems at room temperature involve low energy consumption and are environmentally safe. An advantage of the current method is that synthesized nanoparticles are stable in solution and therefore lend themselves for large-scale process development. pH and temperature are observed to be critical parameters for the synthesis of monodispersed silver nanoparticles [36, 37] and could be extrapolated to the biosynthesis of gold nanoparticles.

Table 1. Zeta potential measurement of the gold nanosuspensions.

Run	Zeta potential of gold nanoparticles (mV)
1	–19.15
2	–11.32
3	–15.88
4	–16.54
5	–18.04
6	–6.45
7	–16.96
8	–26.98
9	–31.79
10	–26.98
Mean	–19.01
Standard error	2.42

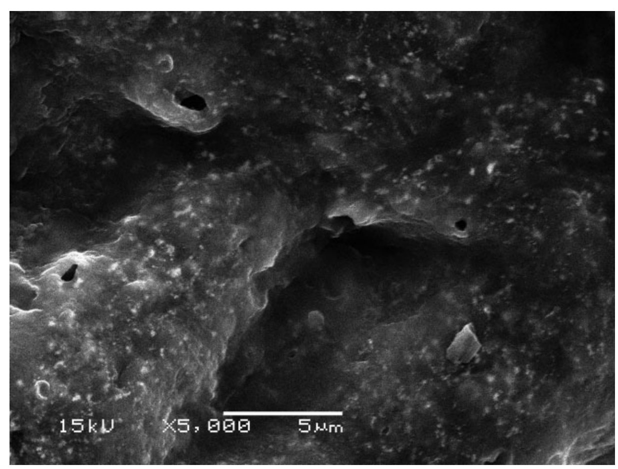

Figure 3. SEM image of gold nanoparticles at 15 kV × 5000 resolution.

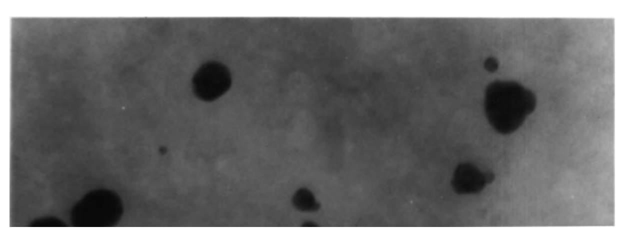

Figure 4. TEM image of gold nanoparticles.

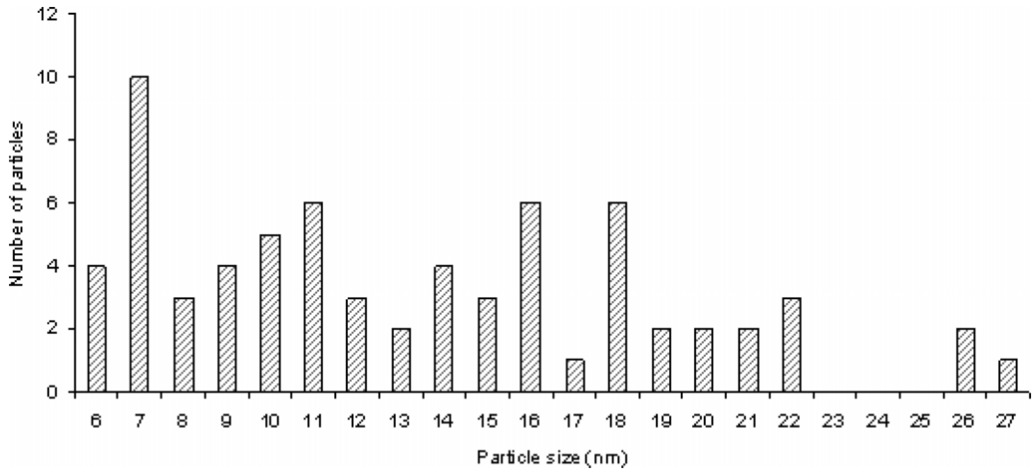

Figure 5. Particle size distribution of gold nanoparticles obtained from TEM image analysis.

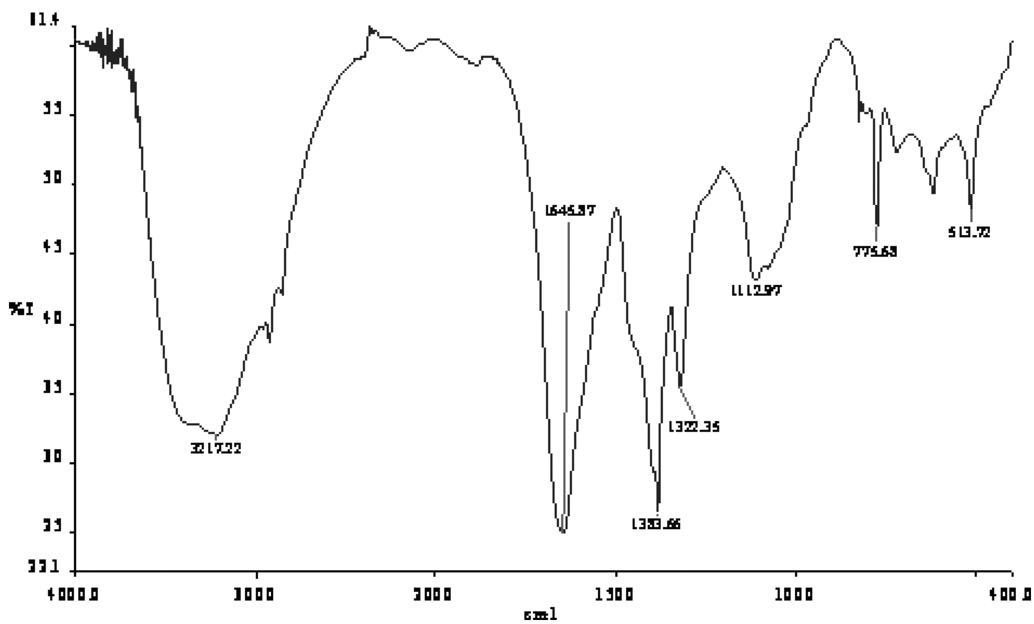

Figure 6. FTIR spectrum recorded from the freeze-dried reaction mixture of gold nanoparticles.

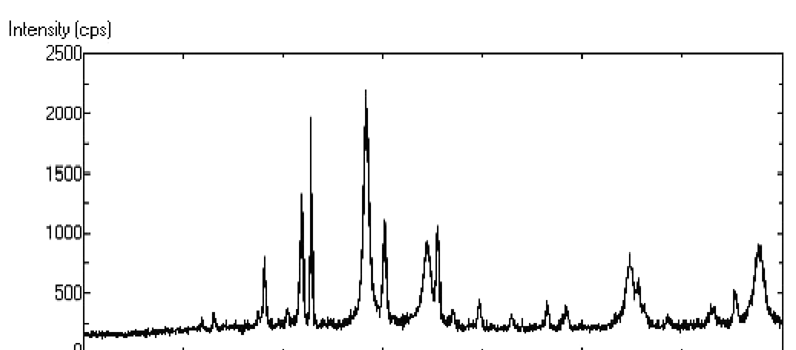

Figure 7. X-ray diffraction pattern recorded from the freeze-dried powder of the gold nanoparticle reaction mixture.

4 Conclusions

The fungal reduction of auric chloride by *Aspergillus niger* yielded gold nanoparticles of 12.79 ± 5.61 nm. The present investigation suggests extracellular biosynthesis and stabilization of gold nanoparticles by proteins secreted in the aqueous filtrate. The advantage of biosynthesis of nanoparticles using this protocol over other methods currently in use is that the nanoparticles are quite stable in solution. This study may therefore lead to the development of a simple bioprocess for the synthesis of gold nanoparticles.

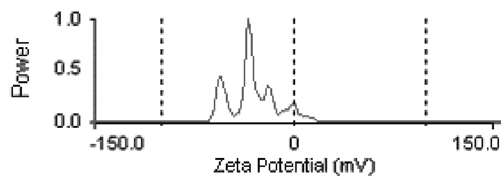

Figure 8. Zeta potential measurement of the gold nanosuspension.

References

[1] M. Sastry, A. Ahmad, M. Khan, R. Kumar, *Curr. Sci.* **2003**, *85*, 162.

[2] N. Vighnehwaran et al.. *Mater. Lett.* **2007**, *61*, 1413.

[3] D. Mandal et al., *Appl. Microbiol. Biotechnol.* **2006**, *69*, 485.

[4] D. Fortin, T. J. Beveridge, in *Biomineralisation: from Biology to Biotechnology and Medical Application* (Ed.: E. Baeuerlein), Wiley-VCH Verlag. Weinheim **2000**.

[5] G. Southam, T. J. Beveridge, *Geochim. Cosmochim. Acta* **1994**, *58*, 4527.

[6] H. A. Lowenstam, *Science* **1981**, *211*, 1126.

[7] D. Pum, U. B. Sleytr, *Trends Biotechnol.* **1999**, *17*, 8.

[8] J. N. Cha et al., *Proc. Natl. Acad. Sci. USA* **1999**, *96 (2)*, 361.

[9] T. Klaus, R. Joerger, E. Olsson, C. G. Granqvist, *Proc. Natl. Acad. Sci. USA* **1999**, *96 (24)*, 13611.

[10] J. C. Chen, Z. H. Lin, X. X. Ma, *Lett. Appl. Microbiol.* **2003**, *37 (2)*, 105.

[11] A. Ahmad et al., *Colloids Surf., B* **2003**, *28*, 313.

[12] Y. Sun, Y. Xia, *Science* **2002**, *298*, 2176.

[13] K. H. Bae et al., *Langmuir* **2006**, *22 (14)*, 6380.

[14] M. I. Husseiny, M. A. El-Aziz, Y. Badr, M. A. Mahmoud, *Spectrochim. Acta Mol., Part A* **2007**, *67*, 1003.

[15] T. J. Beveridge, R. G. E. Murray, *J. Bacteriol.* **1980**, *141*, 876.

[16] M. Sastry, A. Ahmad, M. I. Khan, R. Kumar, in *Nanobiotechnology* (Eds.: C. M. Niemeyer, C. A. Mirkin), Wiley-VCH, Weinheim **2004**.

[17] P. Mukherjee et al., *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 3585.

[18] P. Mukherjee et al., *ChemBioChem* **2002**, *3*, 461.

[19] R. S. Bhambure, *M.Sc. Thesis*, University of Mumbai, India **2008**.

[20] S. Senapati, *Ph.D. Thesis,* University of Pune, India **2005**.

[21] S. Underwood, P. Mulvaney, *Langmuir* **1994**, *10*, 3427.

[22] P. Mulvaney, *Langmuir* **1996**, *12*, 788.

[23] A. Ahmad et al., *Langmuir* **2003**, *19*, 3550.

[24] S. S. Shankar, A. Rai, A. Ahmad, M. Sastry, *J. Colloid Interface Sci.* **2004**, *275*, 496.

[25] J. W. Jeffery, in *Methods in X-Ray Crystallography*, Academic press, New York, **1971**.

[26] A. Gole et al., *Langmuir* **2001**, *17*, 1674.

[27] B. L. V. Prasad et al., *J. Am. Chem. Soc.* **2003**, *125*, 10488.

[28] T. F. Jaramillo, S. H. Baeck, B. R. Cuenya, E. W. McFarland, *J. Am. Chem. Soc.* **2003**, *125*, 7148.

[29] C. Mohr, H. Hofmeister, J. Radnik, P. Claus, *J. Am. Chem. Soc.* **2003**, *125*, 1905.

[30] H. X. Li, L. Rothberg, *J. Anal. Chem.* **2004**, *76*, 5414.

[31] C. M. Goodman, C. D. McCusker, T. Yilmaz, V. M. Rotello, *Bioconj. Chem.* **2004**, *15*, 897.

[32] A. G. Tkachenko et al., *Bioconj. Chem.* **2004**, *15*, 482.

[33] R. T. Tom et al., *Langmuir* **2004**, *20*, 1909.

[34] J. H. Park et al., *Chem. Mater.* **2004**, *16*, 688.

[35] H. Itoh, A. Tahara, K. Naka, Y. Chujo, *Langmuir* **2004**, *20*, 1972.

[36] R. Sanghi, P. Verma, *Bioresour. Technol.* **2009**, *100*, 501.

[37] N. Saifuddin, C. W. Wong, A. A. Nur Yasumira, *E-J. Chem.* **2009**, *6*, 61.

